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High-Affinity HLA-A(*)02.01 Peptides from Parathyroid Hormone-Related Protein Generate In Vitro and In Vivo Antitumor CTL Response Without Autoimmune Side Effects

Guido Francini,2* Antonio Scardino,† Kostas Kosmatopoulos,† François A. Lemonnier,† Giuseppe Campoccia,‡ Marianna Sabatino,§ Daniele Pozzessere,* Roberto Petrioli,* Luisa Lozzi,§ Paolo Neri,§ Giuseppe Fanetti,‡ Maria Grazia Cusi,§ and Pierpaolo Correale*2

Parathyroid hormone-related protein (PTH-rP), a protein produced by prostate carcinoma and other epithelial cancers, is a key agent in the development of bone metastases. We investigated whether the protein follows the self-tolerance paradigm or can be used as a target Ag for anticancer immunotherapy by investigating the immunogenicity of two HLA-A(*)02.01-binding PTH-rP-derived peptides (PTR-2 and -4) with different affinity qualities. PTH-rP peptide-specific CTL lines were generated from the PBMC of two HLA-A(*)02.01+ healthy individuals, stimulated in vitro with PTH-rP peptide-loaded autologous dendritic cells and IL-2. The peptide-specific CTLs were able to kill PTH-rP+HLA-A(*)02.01+ breast and prostate carcinoma cell lines. The two peptides were also able to elicit a strong antitumor PTH-rP-specific CTL response in HLA-A(*)02.01 (HHD) transgenic mice. The vaccinated mice did not show any sign of side effects due to cell-mediated autoimmunity or toxicity. In this study we describe two immunogenic and toxic-free PTH-rP peptides as valid candidates for the design of peptide-based vaccination strategies against prostate cancer and bone metastases from the most common epithelial malignancies. The Journal of Immunology, 2002, 169: 4840–4849.

Cyotoxic T lymphocytes recognize protein Ags as small peptide products of cytoplasmic proteolysis bound to MHC molecules (1, 2). Epitope peptide binding to specific HLA isotypes is determined by definite consensus motifs present in the amino acid sequences of the Ag peptides (1–4).

A number of these sequences have been described for the most common human HLA isotypes. CTL epitope peptides with HLA-A(*)02.01 binding consensus motifs, which are capable of generating Ag-specific CTL lines with antitumor activity, have been derived from the most common tumor-associated Ags (TAA)3 (5–12).

In humans, the large majority of TAA investigated as targets of anticancer vaccine therapy trials are nonmutated self proteins expressed during development (carcinoembryonic Ag (CEA)) and differentiation (tyrosinase, gp100, TRP-1 and -2), or self proteins overexpressed in tumors, such as HER-2/neu, p53, and hTERT (13, 14). All these trials are based on the use of dominant epitopes with a high affinity for HLA, derived from self protein expressed in tumors and in normal tissues. Despite some positive results, increasing evidence indicates that the lack of clear efficacy is probably due to tolerance to self-Ags that concerns dominant determinants for self Ags (15–18).

Experimental evidence demonstrates that the survival and growth of tumor cells in bone tissue is promoted by their interaction with the normal cells involved in bone turnover, and particularly by the abnormal activation of osteoclasts, which makes the bone microenvironment favorable for tumor cell implantation and growth (19–22). A protein produced by tumor cells, designated as parathyroid hormone-related protein (PTH-rP), is mainly responsible for osteoclast cell activation (23). This is a 175- to 177-aa protein that is mainly produced by tumor cells in the bone microenvironment under promotion by TGF-β and other cytokines, whose levels are normally very high in the bone matrix (24, 25). PTH-rP is expressed in 90% of primary prostate and lung spindle cell carcinomas and 50% of primary breast cancers (26–29). It is highly expressed in fetal tissues, where it is involved in bone tissue differentiation (28). Low levels of PTH-rP have been sporadically detected in keratinocytes, uterus, and mammary glands during lactation (29). PTH-rP has functional and amino acid analogies with the parathyroid hormone (PTH) and is capable of acting on the same cell membrane receptor(s) (29, 30).

As a result of these PTH mimetic actions, when PTH-rP is produced in large amounts by tumor cells, it can be responsible for the hypercalcemia of malignancy syndrome (30–32). Because PTH-rP plays a critical role in the occurrence and development of bone metastases, it could be investigated as a biological target for the active TAA-specific immunotherapy of epithelial malignancy bone metastases.

We screened PTH-rP peptides having amino acid sequences with HLA-A(*)02.01 binding consensus motifs and not overlapping the analog peptide sequences of native human PTH.
Two PTH-rP peptides, designated PTR-2 and PTR-4, were chosen for this study and synthesized in the laboratory. The aim of this study was to investigate the ability of PTH-rP peptides to generate peptide-specific CTL lines capable of antitumor activity from HLA-A(*02.01)+ PBMC. Furthermore, the immunological activity as well as the autoimmune and toxic potential of PTH-rP peptides were evaluated in a transgenic HHD mouse model engineered to express human α1 and α2 HLA-A(*02.01 domains (33). The aim of our study was to demonstrate the possibility of raising a CTL-mediated immune response to PTH-rP protein.

Materials and Methods

Peptide synthesis

The peptides (PTR-2 and PTR-4) were synthesized using a solid phase automatic peptide synthesizer (model syto; MultiSynTech, Witten, Germany) and the F-moc/diisopropylcarbodiimide/1-hydroxybenzotriazole strategy. They were cleaved from the resins and detrifield by treatment with trifluoroacetic acid containing ethanediol, water, trisubihyl silone, and anisole (93/2.5/2/L/1). The crude peptides were purified by HPLC using a Vydac C18 column (25 × 1 cm; 10 μm). The products were dissolved in bidistilled water, sterile filtered, and frozen at −70°C at a concentration of 2 mg/ml. Peptide purity was >90% as analyzed by HPLC.

The A2.69 peptide was kindly donated by Dr. J. Schlom (Experimental Oncology Section, National Cancer Institute, National Institutes of Health, Bethesda, MD).

Cell cultures

The LNCap (HLA-A(*02.01)+ prostate carcinoma cell line, the MDA-MB 231 (HLA-A(*02.01)+ breast carcinoma cell line, and the SW1463 (HLA-A(*02.01)+ breast carcinoma cell line) were purchased from the American Type Culture Collection (Manassas, VA). The murine lymphoma cell line EL-4-HHD (HLA-A(*02.01)+) has been previously described (34). The mycoplasma-free cultures were maintained in complete medium (RPMI 1640 for LNCap and EL-4-HHD, and DMEM for SW1463 and MDA-MB 231) (Life Technologies, Grand Island, NY) supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies). The 174CEM-T2 (T2; transport deletion mutant) (35) and CIR-A2 (36) cell lines were provided by Dr. J. Schlom. The T2 and CIR-A2 cells were maintained in IMDM and RPMI 1640 complete medium, respectively.

T2-A2 and HLA up-regulation test

The binding of the PTR-2 and PTR-4 peptides to HLA-A(*02.01) molecules was evaluated by means of flow cytometry using T2 cells and the method described by Nijman et al. (35). In this assay, increased stability (the accumulation of HLA-A(*02.01) molecules on the surface of T2 cells as a consequence of peptide binding) is measured in terms of the increased binding of the Ab directed against HLA-A(*02.01). Briefly, 106 T2 cells in serum-free IMDM were incubated overnight with escalating concentrations (0, 5, 25, and 50 μg/ml) of the PTR peptides in 24-well culture plates at 37°C in an atmosphere containing 5% CO2. The cells were then washed two times with DPBS and subsequently incubated for 1 h with an anti-HLA-A(*02.01 (A2.69) specific mAb (A2.69, catalog no. 189HA-1; One Lambda, Canoga Park, CA), using 10 μl of the recommended dilution per 106 cells. UPC-10 (Cappel/Organon Teknika, West Chester, PA) was used as an isotype control. The cells were again washed two times with DPBS, incubated with 100 μl of a 1/100 dilution of FITC-labeled anti-mouse IgG (BD Biosciences, San Jose, CA), and analyzed by means of FACScan (BD Biosciences) and single-color analysis. The cells were kept at 4°C during all of the manipulations unless otherwise stated.

Generation of DC and T cell lines

PBMCs were isolated from heparinized blood taken from two normal HLA-A(*02.01) volunteers (one male and one female) using a lymphocyte separation medium gradient (Cappel/Organon Teknika) as previously described (37).

The PBMCs (1 × 107 cells/ml) for dendritic cell (DC) enrichment were seeded in T75 flasks in a 10-ml volume of complete medium (RPMI 1640) with 10% FBS for 4 h at 37°C in 5% CO2, after which the nonadherent cells were removed. The adherent cells were then maintained for 7 days in complete medium (RPMI 1640 with 10% FBS, 2 mM glutamine, 100 μg/ml penicillin, and 100 μg/ml streptomycin) containing 50 ng/ml GM-CSF and 0.5 ng/ml IL-4 (both purchased from R&D Systems, Minneapolis, MN). The medium containing GM-CSF and IL-4 was replaced every 48 h. After 7 days of culture, the DC phenotype of the cells was investigated by means of flow cytometry direct immunofluorescence. To estimate DC enrichment before CTL stimulation, the cultures were examined for the expression of CD1a, HLA-I, HLA-DR, CD11c, CD80, CD86, and CD83 markers (38–40).

Cytotoxic assays

Cytotoxic activity of CTLs derived from HHD mice against mouse target cells was tested in 6-h 51Cr release assays. Human CTL activity against human target cells could not be revealed in 6–8 h; 18–h 51Cr release assays were performed as suggested by other authors (6). No explanation currently exists for this observation. It could be due to the partial ability of human tumors to escape the cytolytic effectors, to an intrinsic resistance to the cytolytic mediator released by human CTLs, or possibly to different mechanisms of killing used by human and mouse CTLs.

The human target cells were labeled with 50 μCi of [51Cr]oxyquinoline (Medi Physics, Arlington, IL) for 15 min at room temperature, whereas the mouse EL-4-HHD target cells were labeled with 50 μCi of Na253CrO4 (Amersham, Little Chalfont, U.K.) for 60 min at room temperature. The target cells (0.5 × 104) in 100 μl of complete medium were added to each of the wells in 96-well flat-bottom assay plates (Corning Costar). The labeled targets were incubated at 37°C in 5% CO2 before the addition of effector cells. The T cells were then suspended in 100 μl of AIM-V medium and added to the target cells. The plates were incubated at 37°C for 6 or 18 h, and the supernatants were harvested for gamma counting with harvester frames (Skatron, Sterling, VA). The determinations were made in triplicate and SDs were calculated. All of the experiments were repeated at least three times. Specific lysis was calculated as follows: percentage of specific lysis = (observed release (cpm) − spontaneous release (cpm))/total release (cpm) − spontaneous release (cpm) × 100. Spontaneous release was determined from the wells to which 100 μl of complete medium were added instead of effector cells. Total releasable radioactivity was obtained after treating the target with 2.5% Triton X-100.

Blocking experiments

For the HLA blocking experiments, UPC-10 (Cappel/Organon Teknika) control mAb or anti-HLA-A(*02.01 (A2.69, catalog no.189HA-1; One Lambda) or HLA class I, A, B, C (W6/32; Scra, Sussex, U.K.), were incubated for 1 h before the cytotoxic assay.

Flow cytometry

The flow cytometric analysis procedure has been previously described (41). The cells were first incubated with primary mouse anti-human mAbs against CD3 (C, CD4, CD8, CD56, CD19, CD1a, HLA-I, HLA-DR, CD11c, CD80, CD86, and CD83 (all purchased from BD Biosciences), class I HLA (W6/32; Scra), and MOPC-21 (Cappel/Organon Teknika). Subsequently, they were stained with a FITC-conjugated goat anti-mouse Ig (1/100 dilution; Kirkegaard & Perry Laboratories, Gaithersburg, MD) and, finally, the samples containing 1 × 106 cells in 1 ml of Ca2+/Mg2+-free DPBS were analyzed using a BD Biosciences FACScan equipped with a blue laser with an excitation level of 15 nW at 488 nm. The data gathered from...
10,000 live cells were used to evaluate the results. The procedure for dual-color flow cytometry analysis closely resembles the single-color procedure with the following exceptions: the mAbs were anti-CD3 and anti-CD4 FITC conjugate, anti-CD56 and anti-CD8 PE conjugate, anti-IgG1 FITC conjugate, and anti-IgG3 PE conjugate (BD Biosciences); the cells were simultaneously stained for 1 h, after which they were washed three times, resuspended as above, and immediately analyzed using a BD Biosciences FACScan equipped with a blue laser with an excitation of level of 15 nW at 488 nm and the Lysis II program.

**HLA typing**

The PBMC HLA of the two donors (98/003263 and 98/0033668) was phenotyped by the Blood Bank of the Azienda Ospedaliera Senese (Policlinico S. Chiara, Siena, Italy) using a standard Ab-dependent microcytotoxicity assay and a defined panel of anti-HLA antisera for HLA class I determinations. The polymerase chain reaction was used to type HLA class II.

**Animals**

The HHD mice have been previously described (33). They are αm−/− and D8−/− and express a HLA-A*02.01 monochain consisting of the α1 and α2 domains of HLA-A*02.01, and the α3 domain of DP, linked by its N terminus to the C terminus of human αm by a 15-aa peptide. The mice were housed in a temperature-controlled, light-cycled room. All of the in vivo experiments were performed in accordance with local ethical guidelines.

**Vaccination of HHD mice**

The HHD mice were injected at the base of the tail with 100 μg of the epitope of interest emulsified in IFA in the presence of 140 μg of the I-Aβ-restricted hepatitis B virus core Ag-derived Th epitope (128–140; sequence TTPAYRPNAPIL). The injection was repeated for an immunological boost after 11 days. Two weeks after peptide reboosing (25 days since the first injection), the HHD animals were sacrificed for histology study and in vitro spleen cell restimulation.

**In vivo study of HHD mice injected with PTH-rP peptides**

Two series of three mice were immunized with each one of the two PTH-rP peptides or a control peptide (hTRT). Two series of three animals for each peptide were used at different times. After 11 days, the mice were rechallenged with cognate peptide for reboost. On days 11 and 25, the sera of the immunized HHD mice were collected from the retroorbital sinus for serum Ca ion level evaluation.

Human synthetic thyreocalcitonin (0.1 mg in 1 ml of 0.9% saline solution, catalog no. T3535; Sigma-Aldrich) injected s.c. in the dorsal neck was reinjected after 11 days. Two different CTL lines were generated from each donor and designated T-Donor-1 or T-Donor-2 followed by the specific peptide sequences included in the mature PTH-rP molecules and responsible for its biological activity (amino acids 37–177) (30). The two representative peptides selected for this study were designated PTR-2 (p59–68, FLHH LIAEIH) and PTR-4 (p165–173, TSTTSLELD). PTR-2 was found having the highest HLA-A*02.01 binding affinity, whereas PTR-4, although having noncanonical anchor motifs and an intermediate-low binding affinity, has been previously shown to be very immunogenic (46). Both PTR-2 and PTR-4 showed 100% amino acid divergence from the homolog sequence in the PTH (Table I).

The results presented in the Table I also suggest that PTR-2 and PTR-4 are capable of binding HLA-A*02.01 molecules. A CEA-derived peptide (CAP-1), YLSGANLN, was used as a positive control (Table I).

**Generation of a PTH-rP plasmid/influenza virosomes**

The PTH-rP gene was amplified from the prostate carcinoma DU-145 cell line by RT-PCR (42), starting from the specific mRNA using the sense primer 5′-TTGAGATCCATGCAGGAGACTGGTT-3′ and the antisense primer 5′-CCGAAATTCTCAATGCCTCCGTGAATCGA-3′, and cloned in the BamHI-EcoRI sites of the pcDNA3 expression vector (Invitrogen, San Diego, CA) to obtain the recombinant plasmid GC90. The construct was grown in DHS cells. Plasmid DNA was purified using the Qiagen Endo Free plasmid kit (Qiagen, Valencia, CA) as described by the manufacturer. The influenza virosomes were prepared as described elsewhere (43, 44). The nonencapsulated plasmids were separated by 0.1 gel filtration on a High Load Superdex 200 column (Amersham Pharmacia Biotech, Piscataway, NJ). The column was equilibrated with sterile PBS. The void volume fractions containing the virosomes with encapsulated plasmids were eluted with PBS and collected. For cell transfection, −100 target EL-4-HHD cells were grown in six-well microplates at 37°C and infected with 0.3 μg of DNA virosomes or transfected with 1 μg of plasmid DNA using the Effectene Transfection reagent (Qiagen) as described by the manufacturer. After 2 days, PTH-rP Ag expression was analyzed by evaluating the presence of the specific mRNA by means of RT-PCR and immunofluorescence. Briefly, the cells were washed twice with PBS, fixed with cold methanol/acetone, and treated with a rabbit anti-PTH-rP serum (Calbiochem, La Jolla, CA) followed by FITC-conjugated goat anti-rabbit IgG (1/100). The coverslips were mounted on slides and examined using a Leitz Diplan microscope.

**Statistical considerations**

The between-mean differences were statistically analyzed using StatView statistical software (Abacus Concepts, Berkeley, CA). The results were expressed as the mean value ± SD of four determinations made in three different experiments, and the differences were determined using the two-tailed Student t test for paired samples. A value of p < 0.05 was considered statistically significant.

**Results**

**Peptide selection and HLA-A*02.01 binding**

To test the immunogenicity of PTH-rP protein and the possible tolerance of the human T cell repertoire specific for it, we screened the PTH-rP molecule with the “Parker” algorithm (Bioinformatics and Molecular Analysis Section, Computational Bioscience and Engineering Lab Center for Information Technology) (45) to predict peptides having high theoretical HLA-A*02.01 binding motifs. The functional binding of the two peptides to HLA-A*02.01 molecules was tested in vitro by means of the T2 test. The PTH-rP peptides with similar or overlapping amino acid homology to the analog sequences of PTH were cut out of the screening. The peptide sequences included in the first 36 aa (propeptide) were ignored, although they were theoretically predicted with higher affinity for the HLA-A*02.01 molecule. We considered the peptide sequences included in the mature PTH-rP molecules and responsible for its biological activity (amino acids 37–177) (30). The two representative peptides selected for this study were designated PTR-2 (p59–68, FLHH LIAEIH) and PTR-4 (p165–173, TSTTSLELD). PTR-2 was found having the highest HLA-A*02.01 binding affinity, whereas PTR-4, although having noncanonical anchor motifs and an intermediate-low binding affinity, has been previously shown to be very immunogenic (46). Both PTR-2 and PTR-4 showed 100% amino acid divergence from the homolog sequence in the PTH (Table I).

The results presented in the Table I also suggest that PTR-2 and PTR-4 are capable of binding HLA-A*02.01 molecules. A CEA-derived peptide (CAP-1), YLSGANLN, was used as a positive control (Table I).

**Generation of PTH-rP peptide-specific CTL lines**

To evaluate the PTH-rP-specific T cell repertoire availability in humans, CTL lines specific for each PTH-rP peptide were generated from PBMC of two healthy HLA-A*02.01+ donors by means of cyclic IVS with peptide-pulsed autologous DC and IL-2. Two different CTL lines were generated from each donor and designated T-Donor-1 or T-Donor-2 followed by the specific peptide used for CTL stimulation (T-Donor-1-PTR-2, T-Donor-2- PTR-2, etc.). All of the lymphocyte cultures were investigated for immunophenotype expression and cytolytic activity after at least six stimulation cycles. Direct double-stain flow cytometry immunofluorescence showed that the CTL lines expressed a CD3+CD8+ (range, 60–90%) and CD4+CD8+ (range, 22–40%) and CD4−CD8+ (range, 60–90%). The specificity of CTL lines was evaluated against PTH-rP peptide-pulsed CIR-A2 (HLA-A*02.01+) target cells. Cytotoxic assays demonstrated the peptide specificity of the CTL lines, which were able to kill target cells only when pulsed with the cognate peptide. The CTL lines were unable to kill the unpulsed target cells or those pulsed with the control CAP-1 peptide (Fig. 1). These results demonstrate that a PTH-rP peptide-specific human CTL repertoire is available and mobilizable.

To investigate the functional avidity of these lymphocytes, we investigated their lytic activity against CIR-A2 target cells pulsed...
with different concentrations of the cognate peptide at the fixed E:T ratio of 25:1. These experiments showed that, in comparison with PTR-2, the PTR-4-specific CTL lines induced 25% of killing in target cells pulsed with lower concentrations of cognate peptide (25 g/ml vs 25 g/ml) (Fig. 2). No differences were instead observed against unpulsed or control peptide-pulsed target cells.

Antitumor activity of the PTH-rP peptide-specific CTL lines

To test the antitumor activity of CTL lines specific for PTR-2 and PTR-4, we chose the MDA-MB 231 (breast) and LNCaP (prostate) HLA-A(*)02.01 tumor cell lines as targets. Cytotoxic assays showed that the PTH-rP peptide-specific CTL lines were able to kill both MDA-MB 231 and LNCaP cells (Fig. 3). In contrast, no lysis was observed against the HLA-A(*)02.01 tumor cell line SW-1463 (colon carcinoma). Furthermore, cytotoxic assays also showed that the CTL-mediated killing of MDA-MB 231 and LNCaP target cells was HLA-A(*)02.01 restricted insofar as the addition of an Ab directed against HLA-A(*)02.01 molecules (A2.69) abrogated the cytotoxic effect (Fig. 3); similar results were also obtained using an anti-class I HLA (Fig. 3) mAb (W6/32) (data not shown). Conversely, the addition of an A2.69 isotype-negative control mAb did not interfere with the process of tumor cell killing (Fig. 3).

Cold competition assay

Cold competition assays were performed to check Ag involvement and haplotype restriction of target cell killing. The CTL cytotoxic assays were performed using T2-A2 cells (unpulsed or pulsed with different peptides including PTR-2 and -4) as cold competitors and, respectively,111 In-loaded MDA-MB 231 and LNCaP cells, respectively, as labeled CTL targets. The results showed efficient competition (measured as a reduction in 111 In release) when cognate peptide-pulsed T2 cells were used as cold competitors of MB-MDA-231 and LNCaP target cells. The CTL-mediated killing of

### Table 1. HLA-A2.1 binding of PTH-rP-derived peptides (HLA-A2.1 class I up-regulation test)

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Position in PTH-rP</th>
<th>Amino Acid Sequence</th>
<th>T2 Binding^b</th>
</tr>
</thead>
<tbody>
<tr>
<td>No peptide</td>
<td>NA</td>
<td>NA</td>
<td>244.7 ± 42.43</td>
</tr>
<tr>
<td>PTR-2</td>
<td>59–68</td>
<td>FLHHLIAEIH</td>
<td>279</td>
</tr>
<tr>
<td>PTR-4</td>
<td>23–31</td>
<td>WLKKQLQDV</td>
<td>ND</td>
</tr>
<tr>
<td>PTR-4 peptide-4</td>
<td>165–173</td>
<td>TSTTSLELD</td>
<td>291</td>
</tr>
<tr>
<td>PTR-2 peptide-4</td>
<td>59–68</td>
<td>FLHHLIAEIH</td>
<td>240</td>
</tr>
<tr>
<td>CAP-1^c</td>
<td>NA</td>
<td>YLSGANLNL</td>
<td>NA</td>
</tr>
</tbody>
</table>

^a Peptide binding was evaluated by means of an indirect immunofluorescence FACScan of 174 CEM-T2 cells reacting with an anti-HLA-A(*)02.01 mAb (A2.69 dilution 1/100) and a secondary FITC-labeled anti-mouse IgG (26).

^b Results are expressed in relative fluorescence values (the control value of 287.116 derived from the average mean intensity of 244.7 plus the SD of 42.43 was chosen as the positive cut-off value).

^c CAP-1, an HLA-A2.1-binding carcinoembryonic Ag-derived peptide, was used as a positive control.

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FIGURE 1. Ability of human PTH-rP peptide-specific CTL lines generated from donor 1 (circles) and donor 2 (squares) to lyse CIR-A2 target cells previously pulsed with the cognate peptide. T cell lines generated using PTR-2 (A) or PTR-4 (B) peptides were tested for their ability to lyse 111 In-labeled CIR-A2 pulsed with PTR-2 or PTR-4 peptides (● and ▲ with bold continuous lines, respectively), the control CEA peptide (CAP-1; ● and ▲ with dashed lines, respectively), or no peptide (○ and □ with dashed lines, respectively). An 18-h assay was performed with the peptides being used at a concentration of 25 μg/ml. The results are expressed as the percentage of specific lysis at different E:T ratios. 111 In release in the presence of culture medium without effectors was <15%. Mean values from triplicate determinations in individual experiments (performed at the 6th, 8th, 10th, and 12th CTL line IVS), with SDs. There was a significant difference (p < 0.05, two-tailed t test) of values when results from cognate peptide-pulsed CIR-A2 target cells and controls were compared (CIR-A2 unpulsed or pulsed with the control peptide).
breast and prostate carcinoma cells was, in fact, mainly abrogated by the addition of cold competitors pulsed with the specific peptide used to generate the CTL line. The addition of cold T2 cells, unpulsed or pulsed with a control HLA-A(⁎)02.01-binding CAP-1 peptide, did not interfere with the CTL-mediated killing of cancer cells (Fig. 4). Fig. 4 also shows the ability of CTL lines to recognize and kill T2 cells exposed for 24 h to the specific peptide used for the CTL line generation. The addition of cold CEA-HLA-A2.1-K562 cells did not interfere with the CTL-mediated target cell killing, showing no competition (data not shown).

Target cell PTH-rP production and cell membrane HLA-A(⁎)02.01 expression were evaluated by immunoradiometric assays (IRMA) (47) and indirect immune fluorescence flow cytometry, respectively (Table II).

Taken together, these results demonstrate that killing of HLA-A(⁎)02.01⁎PTH-rP⁎ tumor lines was a peptide-mediated and HLA-A(⁎)02.01-restricted phenomenon.

Ag-specific CTL in HHD transgenic mice vaccination with PTH-rP peptides

To evaluate the immunogenic potential of PTH-rP peptides in vivo, HLA-A(⁎)02.01 mice (HHD) mice were injected with PTR-2, PTR-4 peptide, and an irrelevant control peptide. PTR-2 and PTR-4 showed 100 and 60% amino acid homology, respectively, with the respective mouse peptide sequences. Twenty-five days after the first peptide injection, the spleen cells were isolated and restimulated in vitro with the cognate peptide for 6 days. The spleen cell cultures were then tested against autologous EL4-HHD target cells pulsed with the cognate peptide or expressing PTH-rP after infection with PTH-rP gene plasmids included in influenza virosomes (GC90V).

The construct GC90V used in this study was formerly generated and characterized in our laboratory (44).

The expression of PTH-rP in the GC90V-infected EL4-HHD tissues chosen because of their reported physiologic expression of PTH-rP peptides injected HHD mice were collected and their potential expression of PTH-rP proteins in amounts that are theoretically detectable in vivo by means of a secondary PTH-rP-directed T cell response. Our study failed to demonstrate any PTH-rP mRNA expression in these tissues, in the control as well immunized with PTR-2 or PTR-4. These T cells were able to kill EL4-HHD pulsed with the cognate peptide (PTR-2 and PTR-4, respectively) (data not shown), and the same cells producing PTH-rP after infection with the GC90V (Fig. 5). Spleen cells derived from mice immunized with PTR-2 and -4 could not kill the same target cells pulsed with the control CAP-1 peptide or pulsed with the PTR peptide not used for mouse immunization (data not shown). Target cell lysis was class I MHC restricted, because CTL activity was abrogated when the anti-HLA-A(⁎)02.01 mAb was added to the cytotoxic assays (Fig. 5), whereas the addition of a negative isotype control mAb (UPC-10) did not affect the killing (data not shown). No cytotoxic activity was detected against parental uninfected EL4-HHD cells (data not shown) or those infected with the virosome including the plasmid backbone pcDNA3 (Fig. 5). The spleen cells derived from HHD mice immunized with the control peptide, or not injected at all, did not give rise to any PTH-rP-specific CTL activity; they had slight lytic activity against the target cells at a high E:T ratio, but it was not specific for PTH-rP or class I MHC restricted (Fig. 5), thus suggesting the presence of a residual NK activity. Taken together, these results suggest that the vaccination of HHD mice with PTR-2 and PTR-4 peptides generates a peptide-specific CTL response capable of recognizing naturally processed peptides on tumor cells expressing PTH-rP.

In vivo study of HHD transgenic mice after vaccination with PTH-rP peptides

To evaluate the tissue-specific toxicity and autoimmunity induced by PTH-rP peptide vaccination, a postmortem histology study of tissues chosen because of their reported physiologic expression of PTH or low levels of PTH-rP was performed 25 days after the first peptide injection. At the end of the vaccination cycle, 4-μm-thick paraffin sections taken from the parathyroid, skin, derma, and bone tissues of PTH-rP peptide injected HHD mice were collected and stained with H&E-safranin. The tissues were chosen because of their potential expression of PTH-rP proteins in amounts that are theoretically detectable in vivo by means of a secondary PTH-rP-directed T cell response. Our study failed to demonstrate any PTH-rP mRNA expression in these tissues, in the control as well
as in the vaccinated group of mice, probably because of the extremely low production of this protein in normal conditions (data not shown).

Histology showed the absence of pathological microscopic lymphocyte infiltration and no abnormal inflammatory tables of stained tissues including the parathyroids (data not shown).

Furthermore, to exclude the possibility that the PTH-rP-directed immune response may cross-react with PTH-expressing cells in the parathyroids or in some way affect the osteoclast activity in the bone tissue, we investigated the effects of PTH-rP peptide vaccination on the Ca\(^{2+}\) turnover of HHD mice. To this end, serum Ca\(^{2+}\) ion levels were evaluated during treatment in blood samples collected 11 and 25 days after the first PTH-rP peptide injection. To exclude a delayed effect, blood samples were also drawn 52 days after the first peptide administration in a different set of animals receiving the same above-described treatments. Control blood samples were collected from HHD mice not injected or injected with an irrelevant peptide.

The results demonstrate absence of serum Ca\(^{2+}\) ion fluctuations in any of the transgenic animals, thus indicating that the vaccination with PTR peptides elicits a PTH-rP-specific CTL response without affecting parathyroid functions or bone osteoclast activity (Table III), and without inducing direct cell-mediated bone tissue damage. Taken together, these results suggest that the injection of PTH-rP peptides in HHD transgenic mice generates a PTH-rP-specific response in vivo without affecting normal tissues.

### FIGURE 3

Ability of human PTH-rP-specific CTL lines to lyse in vitro HLA-A(*)02.01\(^+\) tumor cells capable of producing PTH-rP. T cell lines generated using PTR-2 (A and C) or PTR-4 (B and D) peptides were tested for their ability to lyse \(^{111}\)In-labeled prostate (LNCaP; A and B) and breast carcinoma (MDA-MB-231; C and D) target cells. HLA-A(*)02.01\(^+\) colon carcinoma SW-1463 cells incapable of producing PTH-rP were used as a negative control target. The cytotoxic activity of the T cell lines against LNCaP and MDA-MB-231 cells in fresh medium is represented by filled circles and a bold line for the donor 1 and by filled squares and a bold line for the donor 2. The cytotoxic activity of the T cell lines against LNCaP and MDA-MB-231 cells in the presence of the UPC-10 mAb is represented by filled circles and a continuous line for the donor 1 and by filled squares and a continuous line for the donor 2. The cytotoxic activity of the T cell lines against LNCaP and MDA-MB-231 cells in the presence of mouse anti-human HLA-A(*)02.01 mAb is represented by filled up triangles and a dashed line for the donor 1 and by filled down triangles and a dashed line for the donor 2. The cytotoxic activity of the T cell lines against SW-1463 target cells is represented by filled stars and a bold dashed line for the donor 1 and filled diamonds and a bold dashed line for the donor 2. The target cells (1 \(\times\) 10\(^6\)) were labeled with \(^{111}\)In, incubated for 1 h in the presence of medium containing no Ab, negative control Ab UPC-10 (10 \(\mu\)g/ml), or anti-HLA-A2.69 (1/100 dilution), and then used as targets in 18-h cytotoxic assays. The results are from single experiments with triplicate determinations, expressed as the percentage of specific release at different E:T ratios. This experiment was performed three times with similar results (performed at the 8th, 10th, and 12th CTL line IVS). There were significant differences (\(p < 0.05\), two-tailed t test) between the values obtained from the same T cell line against LNCaP plus UPC-10 vs LNCaP plus A2.69; MDA-MB-231 plus UPC-10 vs MDA-MB-231 plus A2.69; LNCaP plus UPC-10 vs SW1463; MDA-MB-231 plus UPC-10 vs SW1463. No significant differences were found when the same T cell line was tested against LNCaP in fresh medium vs LNCaP plus UPC-10 or MDA-MB-231 in fresh medium vs MDA-MB-231 plus UPC-10. The spontaneous release in these assays in the presence or absence of mAbs without effectors was always <15%.
Discussion

The possibility of eliciting a human CTL-mediated immune reaction against PTH-rP could have an immediate clinical application because this protein is a TAA expressed in the most common malignancies (22) and the majority of epithelial malignancy bone metastases (23).

We studied the capacity of two PTH-rP-derived peptides that bind HLA-A(*)02.01 molecules to elicit a strong antitumor response. The vaccination of HLA-A(*)02.01 transgenic mice with the PTH-rP peptides also showed their in vivo ability to generate an effective PTH-rP-specific CTL response with no parallel signs of cell-mediated normal tissue degeneration. We chose target peptides capable of binding HLA-A(*)02.01 because this HLA haplotype is widely expressed in the human population.

We have previously shown that autologous DC pulsed with PTR-4 can generate a PTH-rP-specific CTL response with antitumor activity against autologous metastatic prostate cancer cells and prostate carcinoma cell lines when used to stimulate in vitro tumor-infiltrating lymphocytes isolated from a HLA-A(*)02.01 patient with metastatic prostate carcinoma (46). This finding was intriguing in relation to the definition of PTH-rP as a valid TAA and to the significance of T cell repertoire available for this
Table II.  HLA-A2.1 molecule expression and PTH-rP production in CTL target cells

<table>
<thead>
<tr>
<th>Target Cells</th>
<th>HLA-A2.1 Expression (%)</th>
<th>PTH-rP Production (pg/ml × 10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIR-A2</td>
<td>98.5 (2.2)</td>
<td>Not detectable</td>
</tr>
<tr>
<td>T2-A2</td>
<td>99.56 (1.22)</td>
<td>Not detectable</td>
</tr>
<tr>
<td>LNCaP</td>
<td>29.8 (3.7)</td>
<td>15.2 (5.5)</td>
</tr>
<tr>
<td>MDA-MB231</td>
<td>60.1 (2.2)</td>
<td>25.1 (3.5)</td>
</tr>
<tr>
<td>SW1463</td>
<td>75 (3.2)</td>
<td>Not detectable</td>
</tr>
<tr>
<td>EL-4-HHD infected with influenza virosomes</td>
<td>98.2 (4.24)</td>
<td>Not detectable</td>
</tr>
<tr>
<td>EL-4-HHD infected with influenza virosomes including PTH-rP gene plasmids</td>
<td>96.3 (6.23)</td>
<td>10.56 (3.6)</td>
</tr>
</tbody>
</table>

HLA-A2.1 expression was evaluated by indirect immunofluorescence using an anti-HLA-A(*)02.01 mAb (A2.69) and an FITC-conjugated goat anti-mouse mAb. The results are expressed as percentages of fluorescent cells. Marker expression was considered negative when <4%. The results are expressed as the percentage of each cell sample reactive with mAb. Routinely, 2–4% of cells are stained when treated with a nonpriming mAb or an isotype-related control mAb. PTH-rP production was evaluated using a sandwich IRMA; values of <1.5 pg were considered negative. Numbers in parentheses represent SD.

protein. The particular sequence of the PTR-4 peptide shows an atypical HLA-A(*)02.01 binding motif. We hypothesized that the lack of the canonic anchor amino acid in position two of the peptide sequence (SP2) and in position 9 (DP9) probably leads to an instability of the MHC molecule that underlies the lack of tolerance for this PTH-rP peptide epitope. To investigate this point, and clarify the possibility of inducing a CTL-mediated immune response using different epitope peptides with potential clinical implications, we also selected another peptide, theoretically with higher-affinity HLA-A(*)02.01 binding motifs, after screening of the PTH-rP amino acid sequences using the HLA peptide binding prediction analysis described by Parker et al. (45). Further reasons for choosing the PTH-rP p59–68 FLHHLIAEIH (PTR-2) peptide for the comparative study with p165–173 TSTSELEPD (PTR-4) were its effective binding to HLA-A(*)02.01 molecules (measured by means of the T2 class I HLA up-regulation test) and the high degree of divergence from the analogous peptide sequences belonging to human PTH.

Our results showed that the CTL lines generated using PTH-rP peptide-pulsed DCs had epitope peptide specificity, HLA-A(*)02.01 restriction, and the ability to kill HLA-A(*)02.01+/PTH-rP-producing cancer cells, indicating that PTR-4 and PTR-2 peptides have corresponding human CTL precursor repertoire with Ag-specific antitumour activity ex vivo. Our hypothesis was that the particular sequence feature of the peptides allows a specific T cell repertoire response against a TAA that is mainly expressed during the fetal life, when it is involved in the chondrocyte differentiation. Very small amounts of PTH-rP have also been detected: in cultured skin cells, in the epithelial layer of skin and respiratory mucosa as a result of inflammatory insults and chronic degenerative diseases, in breast tissue during lactation, and in the uterus during pregnancy (29, 48–50). In our study, PTR-2 had the highest predicted affinity for HLA-A(*)02.01, which was confirmed by functional tests in vitro. Its capacity to generate a strong CTL response in PBMC from diverse sources indicated that PTH-rP is not a completely tolerated protein.

![Image](75x155 to 529x304)
The analogous murine sequences in PTH-rP was 100% for PTR-2 and 60% for PTR-4.

...the dorsal neck, was used as a positive control, which is capable after treatment, hypocalcemia (0.64/11006)

...The sequence homology between the human and murine PTH-rP protein sequences was >90%. Amino acid sequence homology between the human PTH-rP peptides and the analogous murine sequences in PTH-rP was 100% for PTR-2 and 60% for PTR-4.

...Blood sample drawn 11 days after the first peptide administration in three different animals.

...Blood sample drawn 52 days after the first administration in a different set of animals receiving the same treatment administered in the same way.

Our data also suggest that the CTL lines specific for PTR-4 peptide have a greater functional avidity than the PTR-2-specific CTL lines, because they could recognize target cells pulsed with lower concentrations of cognate peptide. The observation that PTR-2 has a higher binding affinity for HLA-A(*)02.01 excludes the possibility that these results reflect MHC/PTR-2 peptide complex instability on the target cells. The equal capacity of PTR-4 to generate antitumor CTL lines from different healthy donors adds further evidence concerning its immunogenic features. Our results demonstrate that it is as immunogenic as PTR-2, and so, given their immunogenic and antitumor capacity, PTR-2 and PTR-4 peptides must be considered two PTH-rP epitopes.

To demonstrate the possibility of raising a CTL immune reaction against PTH-rP protein in vivo, we injected the two peptides into HLA-A(*)02.01 transgenic mice. PTR-2 and PTR-4 show 100 and 60% homology, respectively, with the respective murine PTH-rP peptides. The fact that PTR-4 overlaps only five of the nine amino acids of murine PTH-rP peptide allows this epitope to be used as a positive control. After vaccination and in vitro re-stimulation with PTR-2 and PTR-4, we could collect spleen cells capable of killing PTH-rP-expressing tumor cells and cognate peptide-pulsed syngeneic target cells with no apparent signs of in vivo tolerance toward PTH-rP. Interestingly, histology showed no signs of tissue degeneration or abnormal lymphocyte infiltration in comparison with analogous samples taken from control mice. PTR and PTR-4-CTL activity targets, such as bone and kidneys, also showed no anomalies. On the basis of previously described results and considerations, we can exclude the possibility that the absence of autoimmunity was due to TCR degeneracy or target escape phenomena. PTH is a peptide hormone produced by the parathyroids that has a high amino acid and functional homology with PTH-rP and is involved in the control of calcium metabolism. In this context, an efficient immune reaction to PTH-rP may possibly affect calcium turnover or cross-react with PTH, thus leading to parathyroid damage. Pathologic studies excluded any parathyroid involvement or degeneracy, and the monitoring of serum ionized calcium levels in mice previously vaccinated with PTR-4 peptides detected no anomalies in peripheral blood calcium levels. These data may be explained by the fact that PTR-2 and PTR-4 do not show any homology with the corresponding peptide sequences in the human and murine PTH.

The results from human PBMC and HHD transgenic mice indicate the possibility of raising a CTL-mediated immune response against PTH-rP and that PTR-2 and PTR-4 are immunogenic in vivo and capable of generating a strong antitumor response. In our preliminary study, we were unable to detect PTH-rP mRNA in the examined tissues (data not shown); therefore, we have attributed the absence of autoimmune effects to an Ag presentation that is too weak in the normal tissue cells to be targeted by Ag-specific CTL precursors.

Our results suggest that the two PTH-rP peptides examined in this study may be useful in peptide-based cancer vaccines. It may be useful to perform a further study in which all its of the determinants of PTH-rP can be screened for the amplification of a potential panel of epitopes for immunotherapy. One interesting point is the possibility of directing a large immune response toward PTH-rP for preventive purposes after primary debulking by means of surgery, radiotherapy, or chemotherapy. Tumor cells expressing PTH-rP have greater metastatic potential and could be targeted before their implantation in bone tissue, thus potentially reducing the risk of metastatic spreading.

Our data also suggest that the CTL lines specific for PTR-4 peptide have a greater functional avidity than the PTR-2-specific CTL lines, because they could recognize target cells pulsed with lower concentrations of cognate peptide. The observation that PTR-2 has a higher binding affinity for HLA-A(*)02.01 excludes the possibility that these results reflect MHC/PTR-2 peptide complex instability on the target cells. The equal capacity of PTR-4 to...
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